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PURIFICATION OF 2-HYDROXYISOFLAVANONE DEHYDRATASE FROM THE CELL CULTURES OF *PUERARIA LOBATA*

IN HONOUR OF PROFESSOR G. H. NEIL TOWERS 75TH BIRTHDAY

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Key Word Index—*Keywords*: *Pueraria lobata*; Leguminosae; isoflavone biosynthesis; 2-hydroxy-isoflavanone; daidzein; dehydratase; cell cultures.

Abstract—2-Hydroxyisoflavanone dehydratase, which catalyzes the final step of the formation of the isoflavonoid skeleton, was purified and characterized from yeast extract-elicited cell suspension cultures of *Pueraria lobata*. 2-Hydroxyisoflavanone, the substrate of the dehydratase, is the product of 2-hydroxyisoflavanone synthase, as cytochrome P-450 which catalyzes the hydroxylation step associated with aryl migration of flavanone. The dehydratase was purified to apparent homogeneity for the first time by a seven-step purification procedure. It is a single polypeptide with a molecular weight of 38 kDa, and has an isoelectric point at pH 5.1 and a pH optimum at 6.8. It required no co-factor, and the apparent Michaelis constant for 2,7,4′-trihydroxyisoflavanone was 7.0 mM.

INTRODUCTION

Isoflavonoids belong to a class of flavonoids with a C_6–C_3–C_6 skeleton derived from *p*-coumaroyl CoA and three molecules of malonyl CoA (Figure 1) [1, 2]. They share the early steps of their biosynthesis with the general flavonoid biosynthetic pathway until the branching point at formation of the flavanone Fig. 1. As can be seen, isoflavone biosynthesis consists of three enzymatic reactions, namely chalcone synthase (CHS), chalcone-flavanone isomerase (CHI) and isoflavone synthase. CHI is a very stable enzyme which catalyzes the conversion of chalcones into flavanones. Although the established cell cultures of *Pueraria lobata* produce isoflavones, only CHI activity was detected in cell free preparations under the various assay conditions used . However, the activities of CHS and isoflavone synthase could be detected when the cultures were challenged with elicitors.

The first two enzymes, CHS and CHI, have been extensively studied at the enzyme as well as gene levels [3–5]. In the course of studies on isoflavone biosynthesis in *P. lobata* cell cultures, crude enzyme extracts catalyzed the formation of naringenin chalcone (hydroxy-type chalcone) from *p*-coumaroyl and malonyl CoAs. However, in the presence of a large excess of NADPH the 6′-deoxy-type chalcone, isoliquiritigenin, was formed along with naringenin chalcone, a 6′-hydroxy-type chalcone. Isoliquiritigenin is the common precursor of phytoalexins in legumes such as soybean, pea and *P. lobata* [5]. The branching point reaction of isoflavonoid biosynthesis is a migration reaction of the aryl group (B ring), which migrates from C-2 to the adjacent C-3 to generate the isoflavone skeleton. Isoflavone synthase has been regarded as a key enzyme in the biosynthesis of isoflavonoids and its enzyme activity was first detected in fungal elicitor-treated cell suspension cultures of soybean [6]. The reaction mechanism was studied with a microsomal preparation of elicitor-treated *P. lobata* cell cultures and was proven to consist of two step reactions [7, 8]. The enzyme reaction involved in the first step is a cytochrome P-450-dependent monooxygenase which catalyzes the hydroxylation step associated with the aryl migration of flavanone to afford 2-hydroxyisoflavanone. This (P-450) enzyme is now called 2-hydroxyisoflavanone synthase [5, 9] and its reaction mechanism was investigated in detail using ^{18}O labelled isoliquiritigenin and O_2 [5, 8, 10]. On the other hand, the second enzyme that catalyzes dehydration of 2-hydroxyisoflavanone to yield isoflavone was not

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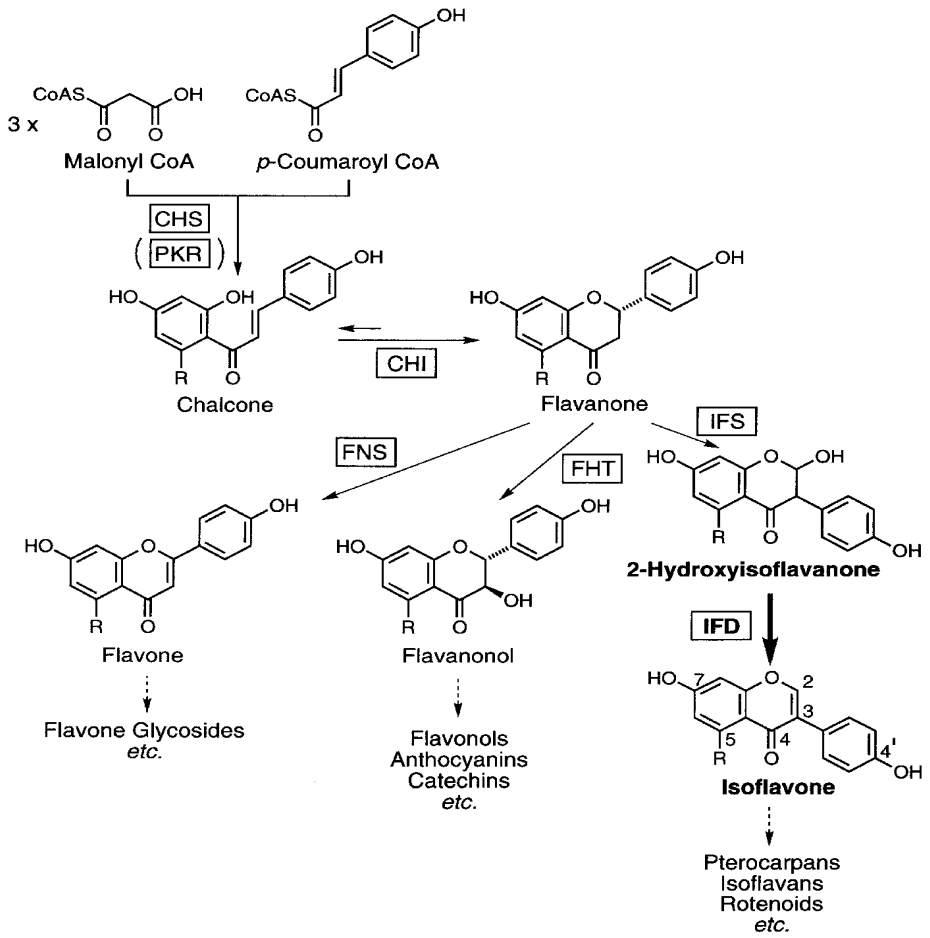

Fig. 1. Biosynthetic pathway of flavonoids and isoflavonoids. CHS = chalcone synthase, PKR = polyketide reductase, CHI = chalcone isomerase, FNS = flavone synthase, FHT = flavanone 3-hydroxylase, IFS = 2-hydroxyisoflavanone synthase, IFD = 2-hydroxyisoflavanone dehydratase. If PKR acts with CHS, R is H, otherwise R is OH. Abbreviations above follow Ref. [8].

fully characterized [5, 7, 8]. The main obstacle for investigation of the dehydratase is the instability of 2,7,4′-trihydroxyisoflavanone, which is the substrate of the enzyme reaction and thus required in the purification and characterization of the dehydratase. This problem was overcome by preparing the unstable 2,7,4′-trihydroxyisoflavanone enzymatically with a microsomal preparation from elicited cell cultures of *P. lobata* and by a rapid enzyme assay method performed with reversed phase HPLC. Accordingly the 2-hydroxyisoflavanone dehydratase (IFD) was purified to apparent homogeneity and some properties of this enzyme were characterized.

RESULTS

Enzymatic synthesis of 2,7,4′-*trihydroxyisoflavanone*

During the course of studies on 2-hydroxyisoflavanone synthase, it was observed that the product of the reaction, 2,7,4′-trihydroxyisoflavanone, was unstable above room temperature and underwent spontaneous dehydration to yield the isoflavone, daidzein; also it can, however, be stored at −80°C without any significant decomposition. It is unstable under basic, rather than acidic, conditions as is expected from its β-hydroxyketone structure. Due to its unstable nature, 2,7,4′-trihydroxyisoflavanone was prepared enzymatically by using microsomal preparations from the yeast extract-treated *P. lobata* cell cultures. When 2-hydroxyisoflavanone dehydratase, was present in microsomal fractions as a contaminant, the yield of 2,7,4′-trihydroxyisoflavanone was remarkably decreased. The microsomal fractions were subjected to repeated ultracentrifugation in order to remove the soluble enzymes, including 2-hydroxyisoflavone dehydratase. The incubation of liquiritigenin with thoroughly washed microsomes in the presence of NADPH gave 2,7,4′-trihydroxyisoflavanone as the main product. 2,5,7,4′-Tetra-

hydroxyisoflavanone was reported to be formed in the reaction of naringenin with a microsomal preparation of soybean cell cultures challenged with elicitor as a byproduct in the formation of the isoflavone, genistein [9]. 2,7,4′-Trihydroxyisoflavanone was first isolated as an intermediate of the isoflavone synthase reaction [10, 11] although physical data for quantification of this compound were not given. Though the NMR spectrum of the enzymatically prepared 2,7,4′-trihydroxyisoflavanone could be measured, it was not sufficient for direct weighing and its amount was determined after its conversion into the isoflavone, daidzein. When 2,7,4′-trihydroxyisoflavanone was incubated with a partially purified dehydratase obtained in a preliminary experiment, daidzein was the only detectable product. The dehydration reaction of 2,7,4′-trihydroxyisoflavanone was, therefore, regarded to proceed quantitatively to afford daidzein, and the amount of 2,7,4′-trihydroxyisoflavanone was quantified as daidzein after enzymatic dehydration. 2-Hydroxyisoflavanone dehydratase solutions of various concentrations were incubated with 2,7,4′-trihydroxyisoflavanone and the resulting reaction mixtures were analyzed by HPLC in order to quantify the peak areas of 2,7,4′-trihydroxyisoflavanone with that of daidzein. Consequently, it was revealed that the absorptivity at 254 nm of daidzein was 6.3-fold higher than that of 2,7,4′-trihydroxyisoflavanone and the quantity of 2,7,4′-trihydroxyisoflavanone was thus determined from the peak area in the HPLC analysis.

Enzyme assay

The protein solution to be assayed was diluted adequately before incubation and the reaction with the purified substrate was carried out for a short period (20 min), because of the limited amount of the substrate was available. The reaction products were determined by reversed phase HPLC. In order to measure many samples as quickly as possible, 60% MeOH was used as a solvent in place of aqueous acetonitrile. A solvent system with water–acetonitrile–acetic acid (71:24:5) was used in previous studies on the isoflavone synthase P-450, where liquiritigenin, 2,7,4′-trihydroxyisoflavanone and daidzein were separated clearly, although daidzein was eluted at more than 60 min [10, 11]. In the case of 60% MeOH the three compounds were all eluted within 15 min. The enzyme activity was calculated from the enzymatically formed daidzein after correcting for the non-enzymatically formed daidzein from control experiments performed in absence of the enzyme.

Purification of the dehydratase

The 2,7,4′-trihydroxyisoflavanone dehydratase was purified about 1200-fold to apparent homogeneity (Table 1). The proteins in the crude extracts were precipitated between 40 and 70% saturation of ammonium sulfate. The concentrated proteins were dissolved in 10 mM potassium phosphate buffer (pH 7.0, 1 mM DTT, 10% glycerol) and desalted on a PD-10 column (Sephadex G-25). In this buffer, 2-hydroxyisoflavanone dehydratase was not retained on a hydroxyapatite column and a large amount of contaminated proteins were removed as bound forms from this column. From the preliminary experiments, when pH of the buffer was changed to pH 6.5, almost half of its enzymatic activity was bound to the hydroxyapatite matrix. In Tris–HCl buffer, as the enzyme was bound to hydroxyapatite, however it was eluted together with other proteins by sodium chloride or ammonium sulfate gradients. After passing through the hydroxyapatite column twice, the unbound fractions were directly loaded onto a DEAE–sepharose column which was eluted by a phosphate gradient (10–150 mM). Active fractions were pooled and applied to a phenyl sepharose column. The chromatography on phenyl sepharose was very efficient to separate from other proteins (Figure 2), although the recovery of the active dehydratase was low. During an ammonium sulfate gradient (20–0%), it was not eluted from the phenyl sepharose column. However, prolonged elution with the final buffer, which contained no ammonium sulfate, released it from the column. Subsequent purification of the enzyme was performed using a Mono Q column chromatography proteins eluted by a sodium chloride gradient (Figure 3). Chromatography on the Mono Q also resulted in a marked increase of specific activity of the enzyme Table 1. The subsequent chromatography on a Mono P column raised the specific enzyme activity only slightly, but significant minor

Table 1. Purification of 2-hydroxyisoflavanone dehydratase from cell suspension cultures of *Pueraria lobata*

Purification step	Volume (ml)	Protein (mg)	Specific activity (mkat/kg protein)	Purification (*x*-fold)	Recovery (%)
1 Crude extract	206	3486	0.048	1	100
2 Hydroxyapatite	350	181.4	1.17	24	125
3 DEAE sepharose	162	35.8	4.43	92	93
4 Phenyl sepharose	26	2.28	13.0	270	17.4
5 Mono Q	1.4	0.31	56.8	1183	10.4
6 Mono P (fraction 18)	0.5	0.06	61.5	1281	2.04
7 G3000SW + G2000SW (fraction 42)	0.2	0.01	56.8	1183	0.33

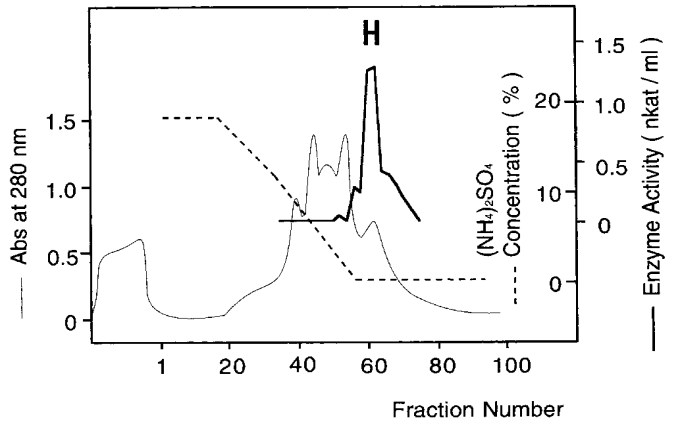

Fig. 2. Hydrophobic interaction chromatography of 2-hydroxyisoflavanone dehydratase of phenyl sepharose. The bar indicates the combined fractions used for subsequent enzyme purification.

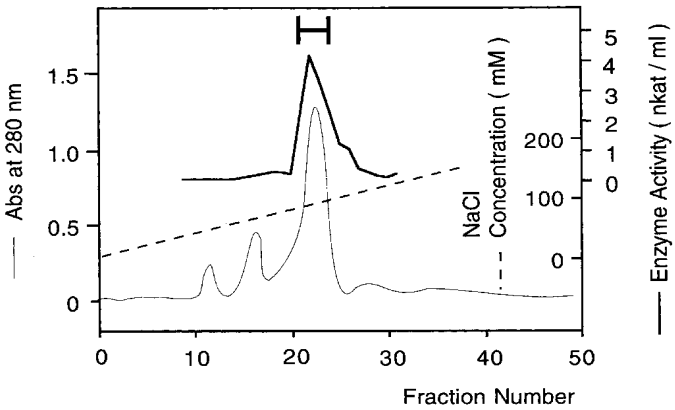

Fig. 3. Ion exchange chromatography of 2-hydroxyisoflavanone dehydratase on Mono Q. The bar indicates the combined fractions used for subsequent enzyme purification.

contaminants were removed from the major peaks (Figure 4). The fraction containing the highest enzyme activity from Mono P was further applied to serially connected TSK G-2000SWXL and G-3000SWXL. After TSK gel filtration chromatography, the fraction containing dehydratase activity appeared as a single band on an SDS–PAGE (Figure 5). The purity of the final fraction was also checked by a reversed phase

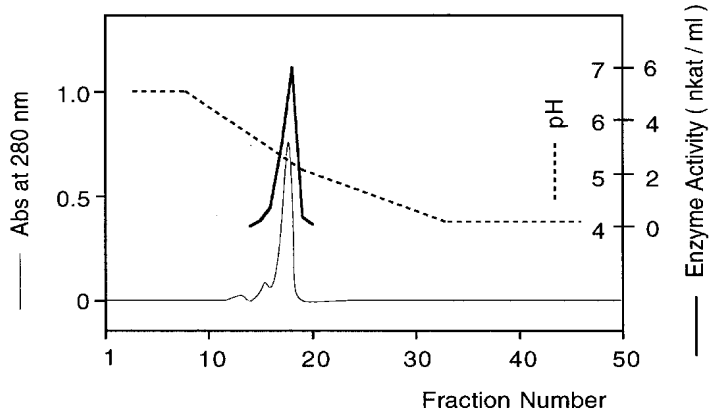

Fig. 4. Chromatofocusing of 2-hydroxyisoflavanone dehydratase on Mono P.

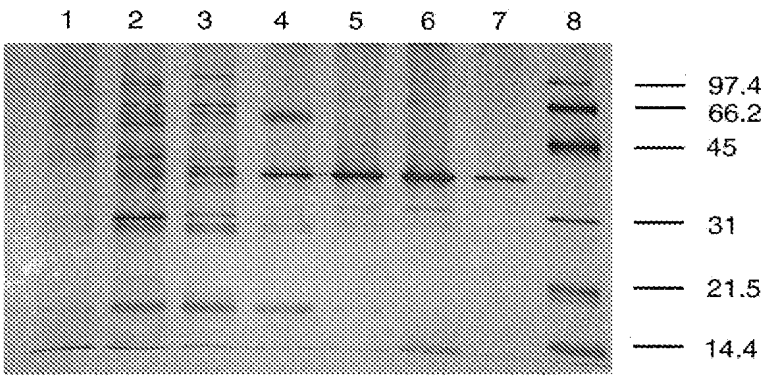

Fig. 5. SDS–PAGE analysis of purification steps for 2-hydroxyisoflavanone dehydratase. Samples were from lane 1, crude extract; lane 2, Hydroxyapatite; lane 3, DEAE sepharose; lane 4, phenyl sepharose; lane 5, Mono Q; lane 6, Mono P (fraction 18); lane 7, TSK gel G3000SW$_{XL}$ + G2000SW$_{XL}$ (fraction 42); lane 8, molecular weight standards.

chromatography on TSK gel phenyl 5PW-RP column which was eluted by an acetonitrile gradient in water where a single peak appeared on the chromatogram when monitored at 280 nm.

Properties of the dehydratase

Its molecular weight was determined to be 38 kDa by comparison of its mobility on the SDS polyacrylamide gel with that of marker proteins. Calibration of the elution volume from serially connected TSK-gels G2000WXL and G3000SWXL gave a nearly identical molecular weight (35 kDa), indicating that it consists of a single polypeptide. The isoelectric point of pH 5.1 was estimated by isoelectric focusing on a Mono P column. The dependence of dehydratase activity on pH was investigated in the pH range from 5.5 to 8.5, and its reaction displayed an optimum at pH 6.8 with half-maximal activity at pH 6.0 and 7.9 in a potassium phosphate buffer. The dehydratase showed no cofactor requirement for its activity and the apparent K_m for 2,7,4′-trihydroxyisoflavanone was found to be 7.0 mM.

Induction of 2-hydroxyisoflavone dehydratase by yeast extracts

Its induction in 7-day-old cell suspension cultures of *P. lobata* after yeast extract treatment is shown in Fig. 6. That is a while before the elicitor treatment weak but significant activity was noted, after challenge with the yeast extract elicitor, its activity showed a transient increase and reached a broad maximum around 10–15 h after addition. In the control experiment, which was performed by the addition of sterile distilled water, no significant change in its activity was observed.

Effect of putative inhibitors

Iodoacetic acid, iodoacetamide and diethyl pyrocarbonate (DEP) which are cystein or histidine-directed reagents, were tested for their inhibitory effects. DEP showed partial inhibition (Table 2). Furthermore, sulfhydryl group-modifying reagents, *N*-ethylmaleimide (NEM) and *p*-chloromercuriphenyl sulfonic acid (PCMP), and arginine-specific reagent 2,3-butadione markedly inhibited its activity at low concentrations.

DISCUSSION

Significantly, the K_m values of the purified dehydratase for 2,7,4′-trihydroxyisoflavanone was low (7.0 mM). Nevertheless, treatment of yeast extract elicitor induced its activity in a very similar manner to that of other enzymes involved in isoflavonoid biosynthesis in *P. lobata* (Hakamatsuka, unpublished results). Therefore, this purified enzyme appears to possess the same physiological function as for other isoflavonoid biosynthetic enzymes but not the non-specific dehydratase, although the dehydration of 2,7,4′-trihydroxyisoflavanone generating daidzein proceeds spontaneously.

Sufficient amount of 2,7,4′-trihydroxyisoflavanone for the dehydratase assay was prepared enzymatically with thoroughly washed microsomes, and the unstable compound was purified by careful reversed phase HPLC column chromatography. This compound was spontaneously dehydrated to yield the 7,4′-dihydroxyisoflavone, daidzein, at $>40°C$, although this conversion also occurs gradually even at room temperature. After its purification by HPLC, it was divided into small portions and stored at $-80°C$ until use, this being stable under these conditions for at least 6 months. By using the enzymatically prepared substrate for the enzyme assay, the dehydratase was purified (for the first time), to apparent homogeneity using yeast extract-treated cell suspension cultures of *P. lobata*. Although its recovery was low, its specific activity was enriched about 1200-fold. Since the peak profiles of enzymatic activity was not divided throughout the six chromatographic steps, this suggests it to

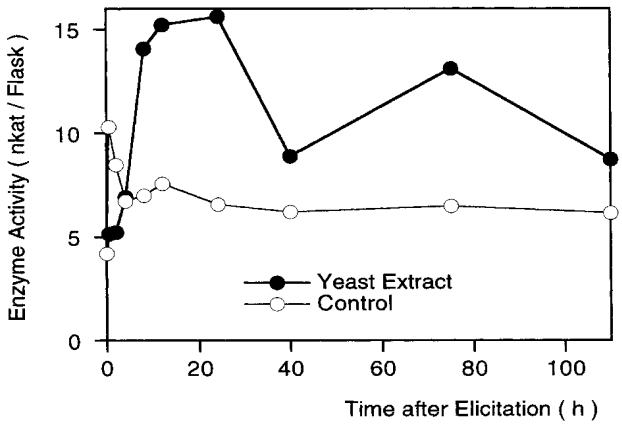

Fig. 6. Induction of 2-hydroxyisoflavanone dehydratase in yeast extract-treated cell suspension cultures of *Pueraria lobata*.

Table 2. Effect of inhibitors on 2-hydroxyisoflavanone dehydratase activity

Inhibitor	Concentration (mM)	Relative activity (%)
None		100
Iodoacetic acid	5	82.1
	50	56.8
Iodoacetamide	5	84.0
	50	44.0
Diethylpyrocarbonate	0.01	84.7
	0.1	52.5
	0.5	31.4
N-ethylmaleimide	0.1	76.8
	1.0	28.9
p-chloromercuriphenyl sulfonic acid	0.001	71.2
	0.01	8.5
	0.1	4.2
2,3-butadione	0.001	84.7
	0.01	27.1
	0.1	0

have only one form in *P. lobata* cells. This contrasts with other enzymes in earlier stages of flavonoid/isoflavonoid biosynthesis, such as phenylalanine ammonia lyase (PAL), chalcone synthase (CHS) and chalcone isomerase (CHI), which consist of several isozymes in various plants [11].

In the phenyl sepharose chromatographic step the dehydratase was eluted with a rather low salt concentration, indicative of its highly hydrophobic nature. This is reasonable to assume since the dehydratase must co-operate with the membrane-bound 2-hydroxyisoflavanone synthase (P-450) which supplies 2,7,4′-trihydroxyisoflavanone. Probably in the living cells the dehydratase is located close to the endoplasmic reticulum or binds loosely to a subcellular particle membrane.

The stereochemistry of the dehydration catalyzed by isoflavone dehydratase is not clear, because the configuration of the 2,7,4′-trihydroxyisoflavanone has not been determined. According to the hypothesis which was proposed for the P-450-dependent oxidative migration of 7,4′-dihydroxyflavanone (liquiritigenin) to give 2,7,4′-trihydroxyisoflavanone, the abstraction of the 3α-proton by the P-450 initiates the reaction; as a result, the conformation of 2,7,4′-trihydroxyisoflavanone released from the P-450 enzyme system is (2*R*, 3*S*) [12]. This proposal is advantageous from a steric viewpoint because the abstraction of the 3α-hydrogen radical, and the migration of the aryl ring, may occur at opposite sides of the pyranone ring. The 3α-hydroxynaringenin isolated and identified as a minor byproduct of the isoflavone synthase reaction also supports the reaction mechanism in which the 3α-hydrogen was abstracted by P-450 (Hakamatsuka *et al.*, unpublished observation) [5]. However, an electronic requirement may prefer

the abstraction of the 3β-proton by P-450 (isoflavone synthase) because most of the oxidations catalyzed by P-450 proceed with retention of configuration. In the former case, the dehydration catalyzed by isoflavone dehydratase must be performed with *syn*-elimination, to which a few examples have been reported, such as the conversion of dehydroquinic acid to dehydroshikimic acid by type I dehydroquinase [13, 14]. In a previous study with ^{18}O labelled liquiritigenin the labelled ^{18}O was completely retained in the product. This excludes the mechanism forming an imine complex, with lysine δ-amino group with C-4 carbonyl, to facilitate dehydration [15]. To clarify the stereochemistry of the P-450-dependent oxidative aryl migration and the subsequent dehydration by IFD, will require studies with (^2H)-flavanones stereospecifically labelled at the 3α- or 3β-positions.

EXPERIMENTAL

Materials

Liquiritigenin (7,4′-dihydroxyflavanone) and daidzein (7,4′-dihydroxyisoflavone) were from our laboratory collection. Bacto yeast extract was from Difco. Hydroxyapatite (Bio-Gel) and molecular weight standards for SDS–PAGE were from Bio-Rad. Sephadex G-25 (PD-10), DEAE sepharose CL-6B, phenyl sepharose HiLoad 26110, Mono Q HR 5/5, Mono P HR 5/5 and Polybuffer 74 were from Pharmacia. TSK gel G2000SWXL, G3000SWXL, phenyl 5PW-RP and ODS-120A were from Tosoh. Molecular weight marker proteins for gel filtration were from Oriental Yeast.

Plant cell cultures and yeast extract treatment

Cell suspension cultures of *Pueraria lobata* were propagated in Murashige–Skoog's medium containing 2 ppm of 2,4-D, 0.1 ppm of kinetin and 3% of sucrose. They were incubated at 25°C in the dark on a rotary shaker and were subcultured every two weeks [7]. For the yeast extract treatment, sterile solution of Bacto yeast extract was added to seven-day-old cultures to make a final concentration of 1 mg/ml. Yeast extract-treated cells were incubated for a further 20–24 h and then harvested via suction filtration. The harvested cells were immediately frozen (liq. N_2) and stored at −80°C until required.

Buffer

The following buffers were used: (A) 100 mM potassium phosphate (pH 7.5) with 14 mM 2-mercaptoethanol and 20% sucrose; (B) 10 mM potassium phosphate (pH 7.0) with 1 mM dithiothreitol (DTT) and 10% glycerol; (C) 10 mM potassium phosphate (pH 6.5) with 1 mM DTT and 10% glycerol; (D) as in C but with 150 mM potassium phosphate; (E) 50 mM potassium phosphate (pH 7.0) with 20% $(NH_4)_2SO_4$, 1 mM DTT and 10% glycerol; (F) as in E but without $(NH_4)_2SO_4$; (G) 10 mM Tris–HCl (pH 7.5) with 1 mM DTT and 10% glycerol; (H) as in G plus 400 mM NaCl; (I) 25 mM BisTris/iminodiacetic acid (pH 7.1); (J) 10% Polybuffer 74/iminodiacetic acid (pH 4.0); (K) 50 mM potassium phosphate (pH 6.8) with 200 mM potassium chloride; (L) 100 mM potassium phosphate (pH 7.0).

Preparation of crude enzyme extract

All operations were carried out at 4°C. The yeast extract-treated cells (100 g) were ground in a chilled mortar with sand (50 g), PVP (10 g) and buffer A (150 ml). The homogenate was filtered through nylon gauze and the filtrate was stirred for 20 min with Dowex I X 2 (20 g) which had been equilibrated with buffer A. The mixture was centrifuged at $2000 \times g$ for 5 min and subsequently at $15\,000 \times g$ for 30 min to remove cell debris and the resin. The supernatant was ultracentrifuged at $105\,000 \times g$ for 60 min to precipitate microsomal proteins which were used for the enzymatic synthesis of 2,7,4′-trihydroxyisoflavanone as described later. The obtained supernatant containing soluble enzymes was fractionated with $(NH_4)_2SO_4$ and the fraction which precipitated between 30 and 70% saturation was dissolved in buffer B (9 ml) and the suspension was homogenized in a glass homogenizer to give a crude enzyme extract. The enzyme extract was desalted by passing through Sephadex G-25 (PD-10) column which had been equilibrated with buffer B. Extraction of crude enzymes was carried out repeatedly according to the method mentioned above. Finally, a total 206 ml of desalted crude enzyme extract was obtained from 1.4 kg of yeast extract-treated cells.

Enzymatic synthesis of 2,7,4′-trihydroxyisoflavanone

The microsomal fraction prepared as described above was again ultracentrifuged at $105\,000 \times g$ for 60 min to wash out contaminating soluble enzymes. Liquiritigenin (0.2 mM) was next incubated with the washed microsomes, in the presence of NADPH (1 mM) at 30°C for 20 min. The incubation mixture was extracted with EtOAc, and the organic layer was dried (Na_2SO_4) and evaporated to dryness under reduced pressure below 40°C. The residue was dissolved in H_2O/acetonitrile/HOAc (71:24:5) and aliquots were repeatedly loaded onto a reversed phase HPLC column (ODS-120A), and eluted with the same above solvent. The peak corresponding to 2,7,4′-trihydroxyisoflavanone from each run was pooled and re-purified by HPLC as before. The purified 2,7,4′-trihydroxyisoflavanone was extracted with EtOAc and aliquots corresponding to about 30 nmol were dispensed into micro tubes, dried under N_2 gas and stored at −80°C until required.

Dehydratase assay

The purified substrate, 2,7,4′-trihydroxyisoflavanone, in a micro-tube (*ca.* 30 nmol) was dissolved in Me_2CO (100 ml) and then mixed with buffer L (3 to 4 ml) which had been chilled on ice. The substrate solution (270 μl) was added to protein samples (30 ml) which were diluted with adequate volume of buffer L prior to incubation. The mixture was incubated for 20 min at 30°C and the reaction was terminated by rapid chilling on ice. The reaction products were extracted with EtOAc (2 ml) and 1.5 ml of organic layer was dried under N_2 gas. The residue was dissolved in 60% MeOH (200 ml) and 20 ml of this solution was analyzed by reversed phase HPLC column (TSK gel ODS-120A, 4.6 × 250 min). The column was eluted with 60% MeOH at a flow rate of 0.8 ml/min and the eluent was monitored at 254 nm. Under these conditions, the retention times of 2,7,4′-trihydroxyisoflavanone and daidzein were 5.2 and 12.5 min, respectively. The control incubation mixture containing 30 ml buffer instead of protein sample was also assayed to measure the non-enzymatic conversion. The amount of daidzein formed was calculated from the peak area using a calibration curve obtained with the standard sample.

Purification of the dehydratase

Hydroxyapatite chromatography. The desalted crude enzyme preparation (206 ml) was applied to a column (5 × 7.6 cm) of hydroxyapatite (Bio Gel), which had been equilibrated with buffer B. Unbound protein fractions eluted with buffer B were pooled (350 ml) and precipitated with $(NH_4)_2SO_4$ (80% saturation) the pellet homogenized in buffer B and desalted on PD-10 columns pre-equilibrated with buffer B. The desalted protein was applied to a second hydroxyapatite column (5 × 4.6 cm) which had been equilibrated with buffer B, and unbound fractions eluted with the same buffer were pooled (150 ml).

Anion-exchange chromatography on DEAE sepharose. The pooled dehydratase-containing fractions from the hydroxyapatite column were adjusted to pH 6.5 with 1 N HCl, and loaded onto a column of DEAE sepharose CL-6B (3.4 × 16 cm) which had been equilibrated with buffer C. The column was washed with buffer C (360 ml) and the dehydratase was eluted by a linear gradient (1200 ml) of 10–150 mM Pi in Buffer C at a flow rate of 1.2 ml/min. Fractions of 10 ml were collected and assayed for dehydratase activity.

Hydrophobic interaction chromatography on phenyl sepharose. The pooled fractions from the DEAE sepharose (154 ml) were brought to 20% saturation with $(NH_4)_2SO_4$, adjusted to pH 7.0 (2 N KOH), and applied to a HiLoad column 26/10 of phenyl sepharose pre-equilibrated with buffer E and the column was washed with 50 ml of buffer E. The dehydratase was eluted by a linear gradient (500 ml) of 0–100% buffer F in buffer E and subsequently with buffer F only (250 ml) at a flow rate of 3 ml/min. Fractions of 9 ml were collected.

Anion-exchange chromatography on mono Q. The pooled enzyme from phenyl sepharose (26 ml) was applied to a PD-10 column pre-equilibrated with buffer G and proteins were eluted with buffer G. Using a sample applicator [Superloop 50 (Pharmacia)], the protein fractions were applied to a Mono Q column (HR 5/5) which had been equilibrated with buffer G. After washing the column with 7 ml of buffer G, the dehydratase was eluted with a linear gradient (42 ml) of 0–40% buffer H in buffer G at 0.7 ml/min and fractions of 0.35 ml were collected.

Chromatofocusing on mono P. Using a PD-10 column, the buffer of the pooled fractions from Mono Q (1.4 ml) was changed to buffer I, and the protein fractions were applied to a Mono P column (HR 5/5) which had been equilibrated with buffer I. The column was washed with 5 ml of buffer I and the proteins were eluted with 30 ml of buffer J at 1 ml/min which generated a pH gradient from 7.1 to 4.0, and fractions of 0.5 ml were collected and measured for their pH value.

Gelfiltration chromatography on G2000SWXL and G3000SWXL. An aliquot (0.25 ml) of the dehydratase containing fractions from Mono P was applied in tandem to G2000SWXL and G3000SWXL columns which had been equilibrated with buffer K. The proteins were eluted with buffer K at 0.4 ml/min and 0.2 ml fractions were collected. Furthermore, a mixture of molecular weight standard proteins (290 000, glutamate dehydrogenase; 142 000, lactate dehydrogenase; 67 000, enolase; 32 000, adenylate kinase; 12 400, cytochrome C) were chromatographed under the same condition to calibrate the column system.

Analytical methods

Protein concentration was estimated by the Bio-Rad protein assay using bovine serum albumin as standard. SDS–polyacrylamide gel electrophoresis was performed according to Laemmli [10] with 12% gel and proteins were silver stained. Molecular weight standards for SDS–PAGE (Bio-Rad) (rabbit muscle phosphorylase b, 97 400; bovine serum albumin, 66 200; hen egg white ovalbumin, 45 000; bovine carbonic anhydrase, 31 000; soybean trypsin inhibitor, 21 500; hen egg white lysozyme, 14 400), were used for molecular weight calibration.

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REFERENCES

1. Dewick, P. M., In *The Flavonoids, Advances in Research since* 1980, eds J. B. Harborne and T. J.

Mabry. Chapman and Hall, London, 1988, pp. 535–640.
2. Hakamatsuka, T. and Sankawa, U., *J. Plant Res. Special Issue*, 1993, **3**, 129.
3. Heller, W. and Forkmann, G., in *The Flavonoids, Advances in Research since* 1986, ed. J. B. Harborne. Chapman and Hall London, 1994, p. 499–536.
4. Forkmann, G. in *The Flavonoids, Advances in Research since* 1986, ed. J. B. Harborne. Chapman and Hall London, 1994, p. 537.
5. Sankawa, U. and Hakamatsuka, T., in *Dynamic Natural Products*, eds K. Ogura and U. Sankawa. Kodansha Scientific, Tokyo, 1997, pp. 25–48.
6. Hagmann, M. and Grisebach, H., *FEBS Lett.*, 1984, **175**, 199.
7. Hakamatsuka, T., Noguchi, H., Ebizuka, Y. and Sankawa, U., *Chem. Pharm. Bull.*, 1989, **37**, 249.
8. Hakamatsuka, T., Noguchi, H., Ebizuka, Y. and Sankawa, U., *Chem. Pharm. Bull.*, 1942, **1990**, 38.
9. Kochs, G. and Grisebach, H., *Eur. J. Biochem.*, 1986, **155**, 311.
10. Hashim, M. F., Hakamatsuka, T., Ebizuka, Y. and Sankawa, U., *FEBS Lett.*, 1990, **271**, 219.
11. Hakamatsuka, T., Hashim, M. F., Ebizuka, Y. and Sankawa, U., *Tetrahedron*, 1991, **47**, 5969.
12. Heller, W. and Forkmann G., in *The Flavonoids, Advances in Research since* 1980, ed. J. B. Harborne. Chapman and Hall, London, 1988, p. 499.
13. Laemmli, U. K., *Nature*, 1970, **227**, 680.
14. Kleanthous, C., Deka, R., Davis, K., Kelly, S. M., Cooper, A., Harding, S. E., Price, N. C., Hawkins, A. R. and Coggins, J. R., *Biochem. J.*, 1992, **282**, 687.
15. Vaz, A. D. N., Bulter, J. R. and Nugent, J., *J. Am. Chem. Soc.*, 1975, **97**, 5914.